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14. ABSTRACT

Taking advantage of the Connectivity Map database that contains gene signatures for 1309 drugs we tested the hypothesis that drugs that elicit gene signatures most opposed to metastatic prostate cancer will be effective anti-cancer drugs. Our data provide strong evidence that gene expression profiles can be utilized to predict which drugs may be effective in killing hormone-refractory PCa cells. We have identified a metastatic PCa-specific gene signature by identifying a set of genes commonly deregulated in at least 4 published datasets of metastatic PCa. We have demonstrated that the Connectivity Map database can be exploited to identify potential anti-PCa drugs that can be tested in cell culture and eventually in animal models for preclinical validation. Since the drugs that we selected are FDA approved, clinical trials could be rapidly initiated if the animal experiments are successful. 5 out of 11 drugs, selected as high scorers in the Connectivity Map analysis of metastatic PCa, are indeed potent inducers of apoptosis in PCa cell lines, and 3 of them demonstrate enhanced efficacy in killing of hormone-refractory PCa cells indicating that this set of structurally unrelated drugs indeed elicits anti-PCa activity. These drugs will be tested in PCa mouse models for anti-tumor activity.

15. SUBJECT TERMS
prostate cancer, Connectivity Map database, drugs, transcriptional profiling, gene expression, gene signature, treatment, apoptosis

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Final Report
INTRODUCTION:
Despite progress in treating prostate cancer (PCa), most men with hormone-refractory, metastatic disease eventually develop resistance to therapy and succumb to the disease. In an attempt to remedy this situation, we proposed to use knowledge from PCa gene profiling studies to identify molecular pathways amenable to "targeted" therapies. Several transcriptional profiling studies have identified PCa specific gene signatures and classified chromosomal abnormalities. Recently, transcriptional profiling of various human cell lines treated with 1309 bioactive small molecules led to the establishment of the Connectivity Map (CMAP) database of more than 7000 genome-wide expression profiles which can be used to identify gene signatures in patient samples that closely resemble specific treatment gene signatures. The hypothesis to be tested was that PCa-specific gene signatures can be linked to particular drug-associated gene signatures in the CMAP database. We proposed to identify and validate new drugs against PCa, based on the premise that drugs that revert the PCa gene signature towards a "normal prostate" gene signature will have a strong anti-cancer effect. Taking advantage of the CMAP database we proposed to test the hypothesis with drugs that elicit gene signatures most opposed to PCa. These drugs were to be tested for their abilities to reverse the hormone-refractory PCa phenotype towards a gene expression profile similar to normal prostate. Drugs that reverse the PCa phenotype by affecting PCa-relevant gene signatures would then be tested for their ability to kill PCa cells in culture. Finally, the drugs would be tested in a unique genetic mouse model of PCa, ERG/PTEN transgenics, that closely emulates human PCa. Thus, our aims were:

Aim 1. Apply PCa gene signatures to the CMAP database in order to identify drugs that are expected to reverse PCa specific gene expression profiles
**Aim 2. Test the highest scoring drugs from the CMAP analysis in PCa cell lines and animal models**

BODY:

Task 1. **Apply prostate cancer gene signatures to the Connectivity Map database in order to identify drugs that are expected to reverse prostate cancer specific gene expression profiles**
1a. **Published prostate cancer transcriptional profiling data including benign prostate, primary prostate cancer and hormone-refractory prostate cancer metastasis gene signatures as well as ERG translocation positive and ERG translocation negative gene signatures will be selected and meta-analysis will be performed to identify a common set of genes that is specific for hormone-refractory prostate cancer (Hormone-refractory prostate cancer specific gene signature).**

Data collection, pre-processing of microarray data, and strategy for discovery of the metastatic PCa gene signature

To overcome the limitations of individual microarray analysis, meta-analysis of the data from multiple gene expression profiling studies can be used to increase statistical power and, thereby, i) identify potentially important genes that may be missed in a single study alone, ii) weed out possible false positives from individual studies, iii) develop a more reliable genetic signature. Furthermore, meta-analysis approaches that
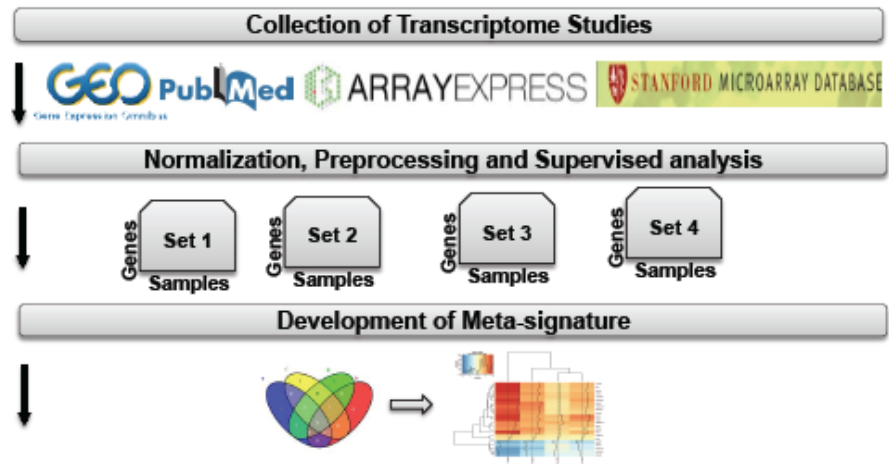
incorporate reanalysis of original data from multiple microarray studies (as opposed to the conventional approach of comparing the interpreted results of separate studies), generate gene signatures with increased reproducibility and sensitivity, thus revealing biological insight not evident in individual datasets. Given the limitation of microarray gene expression data generated from any single PCa profiling studies to translate into consistent gene expression changes, we hypothesized that a meta-analysis strategy to combine multiple microarray datasets will uncover a gene expression signature that facilitates identification of novel drugs for PCa. To discover a common PCa gene signature we distilled the plethora of potential PCa gene expression changes through an optimized meta-analysis strategy that included normalization and statistical testing procedures on the raw data from four independent PCa transcriptome datasets as well as separate analyses on the individual datasets using identical analysis parameters.

We searched the literature and publicly available microarray repositories for gene expression studies of human PCa specimens. We collected 4 datasets that include samples of normal prostate, primary PCa, and metastatic PCa for development of a metastatic PCa gene signature. All datasets utilized either Affymetrix GeneChip arrays, the Agilent platform or Stanford custom microarrays and either whole tissues or microdissected samples. The GEO datasets include i) GSE21034: 218 patients with primary or metastatic PCa and normal adjacent tissue; ii) GSE3933: 62 PCa, 9 lymph node metastases and 41 normal prostate samples; iii) GSE32269: 22 primary PCa and 29 metastatic PCa; iv) GSE8511: 16 benign prostate tissue, 12 local PCa tissue, 13 metastatic PCa tissue.

Data analysis was performed primarily through the use of R packages implemented in Bioconductor. Each dataset was normalized from raw data using the Frozen RMA (fRMA) algorithm. To minimize bias introduced by the range of methodologies, we applied the same global normalization and filtering approach to each dataset and subsequently implemented a consistent procedure for differential gene expression analysis across the datasets. The normalized datasets were preprocessed using Z-statistics normalization to reduce batch effects among different datasets. The empirical Bayes moderated t-statistic identified differentially expressed genes. The differentially expressed genes were selected using a very stringent Benjamini and Hochberg method for multiple comparison correction to control for FDR. The individual analysis of each dataset resulted in 4 lists of differentially expressed genes.

Development of the metastatic PCa gene signature

We generated a list of differentially expressed genes for each dataset using empirical Bayes moderated t-statistic and selected the top ranking genes that distinguish metastatic PCa from primary PCa and normal prostate. Moreover, we used very stringent criteria to select the top ranking genes that distinguish metastatic PCa from primary PCa and normal prostate: each gene had to be differentially expressed in at least 3 of 4 microarray studies, yielding a false discovery rate (FDR) of <10%. Gene specific statistical significance comparison across studies identified genes consistently differentially expressed in metastatic PCa compared to primary PCa and normal prostate. Venn diagram analysis of the differentially expressed

genes of the four datasets identified 376 genes common with concordant directionality to at least 3 of the 4 datasets. These genes were used for analysis in the C-Map database.

This task took longer to accomplish than originally expected, since we had to establish the specific algorithms and modifications of the analytic strategy in order to accomplish this task.

1b. **The hormone-refractory prostate cancer specific gene signature established in Task 1a as well as available gene signatures specific for the ERG/PTEN mice and the LNCaP CL1 vs. LNCaP gene signature will be screened against the 7000 gene signatures for 1309 bioactive small molecules in the Connectivity Map database in order to identify and rank small molecules that reverse the hormone-refractory prostate cancer specific gene signature and reverse the ERG/PTEN prostate cancer gene signature.**

Identification of candidate small molecule drugs in the C-Map database that are anticipated to reverse the metastatic PCa gene signature
We used the 376 gene metastatic PCa expression signature to interrogate the C-Map database in order to identify compounds that were anti-correlated with the metastatic PCa-specific signature. C-Map database analysis identified compounds that were anti-correlated with the metastatic PCa-specific signature, i.e. induced a gene expression pattern consistent with non-metastatic prostate. These small molecules were ranked according to their inverse geneset enrichment score. The top 50 drugs are listed in Table 1.
The available ERG/PTEN and LNCaP CL1 vs. LNCaP gene signatures did not add any additional necessary useful information since these data were derived from cell lines and a transgenic mouse model and, therefore, we decided to focus only on the common set of genes of the different human patient sample gene signatures for further evaluation at this point.

1c. **The 10 FDA approved small molecules with the highest ranking scores of inverted gene signatures that are also high ranking in the ERG/PTEN analysis will be selected for further evaluation and validation in *in vitro* and *in vivo* assays. Primary focus for this proposal will be on FDA approved drugs.**

For further evaluation of the potential efficacy of the highest ranking small molecules to affect PCa cells, we first focused among the highest ranking small molecules on FDA approved drugs with the notion that if we can demonstrate efficacy of a FDA approved drug, clinical translation would be much faster than if we focus on experimental small

TABLE 1

cmap name	enrichment
decitabine	-0.998
(-)-catechin	-0.964
sanguinarine	-0.944
5152487	-0.942
5213008	-0.942
sulfadoxine	-0.897
scopoletin	-0.844
oligomycin	-0.826
ursodeoxycholic acid	-0.822
dopamine	-0.815
tioguanine	-0.811
5186223	-0.797
BW-B70C	-0.793
topiramate	-0.789
STOCK1N-35874	-0.78
phenanthridinone	-0.774
convolamine	-0.768
amrinone	-0.766
bacitracin	-0.762
tomelukast	-0.762
15(S)-15-methylprostaglandin E2	-0.757
5162773	-0.75
5230742	-0.746
azapropazone	-0.745
arachidonyltrifluoromethane	-0.741
Prestwick-1083	-0.741
decamethonium bromide	-0.739
praziquantel	-0.739
bisoprolol	-0.737
5666823	-0.727
amphotericin B	-0.725
5182598	-0.725
tiaprofenic acid	-0.72
canavanine	-0.71
DL-PPMP	-0.706
diflorasone	-0.702
sulindac sulfide	-0.702
carmustine	-0.692
sotalol	-0.689
(-)-atenolol	-0.686
STOCK1N-35696	-0.684
racecadotril	-0.672
MG-132	-0.67
penbutolol	-0.669
zalcitabine	-0.667
doxepin	-0.666
acenocoumarol	-0.663
tyrphostin AG-1478	-0.663
apramycin	-0.662
5186324	-0.661

molecules not approved for human use. We selected the following 11 drugs (highlighted in Table 1) for further evaluation: decitabine, sulfadoxine, oligomycin, ursodeoxycholic acid, tioguanine, topiramate, amrinone, bacitracin, azapropazone, praziquantel, sulindac sulfide.

Task 2. **Test the highest scoring drugs from the Connectivity Map analysis in prostate cancer cell lines and animal models**
      2a. **The 10 highest scoring FDA approved small molecule drugs from Task 1 at physiologically achievable doses will be tested in hormone-responsive (LNCaP, VCaP) and hormone-refractory (CL1, PC-3, DU145) prostate cancer cell lines for their abilities to inhibit cell proliferation and soft agar anchorage-independent growth and to induce cell cycle arrest or apoptosis using high throughput liquid handling robots and 96 well plates.**

Most of the high scoring candidate drugs induce apoptosis in RCC cell lines

Eleven of the high scoring drugs for metastatic PCa were tested with regard to their effects on survival of the hormone-refractory PCa cell line, PC-3. 5 (decitabine, oligomycin, topiramate, praziquantel, sulindac sulfide) out of 11 evaluated drugs strongly induced apoptosis relative to the solvent DMSO when used at the concentration applied in the C-Map database indicating that this set of structurally unrelated drugs indeed elicits anti-PCa activity. To determine the dose response for each of the drugs we performed a dose response curve in these cells for the 5 apoptosis inducing drugs (Fig. 1). Figure 1 shows that each of the 5 drugs induced apoptosis efficiently at doses that are physiologically relevant. Similar results were obtained in DU145 cells.

PC-3	Dose	value
#		
Decitabine	10micromolar	**0.973**
Decitabine	5micromolar	**0.773**
Decitabine	2micromolar	**0.564**
Decitabine	1micromolar	**0.335**
Olygomycin	1micromolar	**0.934**
Olygomycin	0.5micromolar	**0.682**
Olygomycin	0.2micromolar	**0.574**
Olygomycin	0.1micromolar	**0.401**
Topiramate	10micromolar	**0.754**
Topiramate	5micromolar	**0.483**
Topiramate	2micromolar	**0.391**
Topiramate	1micromolar	**0.349**
Praziquantel	1micromolar	**0.789**
Praziquantel	0.5micromolar	**0.597**
Praziquantel	0.2micromolar	**0.411**
Praziquantel	0.1micromolar	**0.333**
Sulindac sulfide	100micromolar	**0.965**
Sulindac sulfide	50micromolar	**0.893**
Sulindac sulfide	20micromolar	**0.666**
Sulindac sulfide	10micromolar	**0.471**
DMSO	0.50%	**0.345**

Fig. 1. Apoptosis assay for PC-3 cells treated with the indicated doses of drugs that scored high on the Connectivity Map analysis.

Several of the high scoring candidate drugs induce more apoptosis in LNCaP CL1 cells than the parental hormone-responsive LNCaP cells

To determine whether the highest scoring drugs induce differential apoptosis in PCa cells in dependence of hormone-responsiveness the same drugs as above were incubated with the hormone-refractory LNCaP

CL1 cells, a hormone-refractory clone derived from hormone-responsive LNCaP cells, and hormone-responsive LNCaP cells. The 5 drugs that were effective in killing PC-3 cells also strongly induced apoptosis in LNCaP CL1 cells relative to control DMSO and 3 of these 5 drugs (decitabine, topiramate, oligomycin) had a significant reduction in apoptosis induction in parental LNCaP cells indicating a potential correlation with hormone-response and a more advanced and aggressive stage of PCa (Fig. 2).

Drug	Dose	LNCaP Cl1 Apoptosis most	std dev	LNCaP Apoptosis	std dev
Decitabine	5μM	0.975	0.027	0.663	0.102
Topiramate	10μM	0.985	0.019	0.608	0.011
Olygomycin	1μM	0.841	0.035	0.475	0.034
Praziquantel	1μM	0.538	0.013	0.551	0.025
Sulindac Sulfide	50μM	0.921	0.009	0.945	0.031
DMSO	0.50%	0.264	0.022	0.268	0.015

Fig. 2. Apoptosis assay for LNCap CL1 and LNCaP cells, treated with the indicated doses of drugs that scored high on the Connectivity Map analysis.

2b. The 4 most effective drugs will also be tested for synergistic activity in above assays.

Double and triple combinations of low doses of high scoring candidate drugs are highly effective in inducing apoptosis in PCa cells

To determine whether combining multiple drugs at low doses enhances induction of apoptosis in PCa cells we combined the 5 top scoring drugs at doses that by themselves do not induce apoptosis. As shown in Figure 3, several combinations were highly effective in inducing apoptosis in LNCaP CL1 cells. Particularly, combinations of low doses of decitabine with topiramate or sulindac sulfide enhanced apoptosis significantly. Similarly, triple combinations of low doses of the top 5 drugs induced apoptosis significantly more effectively than either alone or double combinations demonstrating synergistic activity (Fig. 3).

	LNCaP CL1
Topiramate	0.449
Oligomycin	0.414
Decitabine	0.423
Sulindac sulfide	0.322
Praziquantel	0.569
Topiramate + oligomycin	0.484
Topiramate + Decitabine	0.881
Topiramate + Sulindac sulfide	1.124
oligomycin + Decitabine	0.964
oligomycin + Sulindac sulfide	0.431
Decitabine + Sulindac sulfide	1.023
Praziquantel + Topiramate	0.609
Praziquantel + oligomycin	0.852
Praziquantel + Decitabine	0.585
Praziquantel + Sulindac sulfide	0.773
Topiramate + oligomycin + Praziquantel	1.030
Topiramate + oligomycin + Sulindac sulfide	1.844
Topiramate + oligomycin + Decitabine	1.739
Topiramate + Praziquantel + Sulindac sulfide	1.124
Topiramate + Praziquantel + Decitabine	0.901
Topiramate + Sulindac sulfide + Decitabine	1.941
Sulindac sulfide + oligomycin + Praziquantel	1.322
Sulindac sulfide + oligomycin + Decitabine	1.568
Sulindac sulfide + Praziquantel + Decitabine	1.425
Praziquantel + oligomycin + Decitabine	0.935
DMSO	0.356

Fig. 3. Combinations of low doses of 5 Top Scoring Drugs are effective in inducing apoptosis.

2c. **For these proof-of-concept studies anti-tumor efficacy of the 3 lead compounds against prostate cancer, namely compounds that elicit the strongest anti-tumor efficacy in the cell-based assays, will be assessed in two human prostate cancer xenograft mouse models (LNCaP, LNCaP CL1; 25 mice per study, 75 mice total) orthotopically using established efficacious treatment doses for these FDA approved drugs.**

To develop a xenograft model of PCa, we have engineered the LNCaP and LNCaP CL1 expressing firefly luciferase using a retrovirus that encodes a fusion of luciferase and neomycin phosphotransferase (LucNeo) to enable tumor burden quantification using non-invasive *in vivo* imaging. LNCaP and LNCaP CL1 cells were transduced with a VSVG-pseudotyped retrovirus encoding a fusion of firefly luciferase with neomycin phosphotransferase (LucNeo). LNCaP- and LNCaP CL1-LucNeo cells were selected in 1 mg/ml of G418 and demonstrated to express luciferase.

Since the above experiments and the engineering of the cells took significantly longer and more resources than originally anticipated, we have been unable to initiate the animal experiments prior to the end of the funding period. However, we plan with additional resources to implant the LNCaP- and LNCaP CL1-LucNeo cells and followed for tumor growth using *in vivo* bioluminescence imaging (BLI) endpoints. Animals with established tumors of ~200 mm3 will be divided into 3 cohorts to be treated with vehicle, decitabine, topiramate, and oligomycin at maximally tolerated dose (MTD), and at ½ x MTD for 2-3 weeks as detailed in our SOW.

2d. **If *in vitro* testing identifies combinations of drugs with synergistic activity, the 2 most effective combinations will also be tested *in vivo* as above.**

This task was not initiated due to the aforementioned time constraints.

2e. **The same 3 drugs will be tested in the ERG/PTEN prostate cancer model using the same study design and endpoints as in task 2b.**

This task was not initiated as we used resources and time to pursue and finish task 1 and tasks 2a and 2b as well as the first part of 2c.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Meta-analysis of metastatic PCa gene signatures
- Identification of key genes deregulated in metastatic PCa
- Identification of candidate small molecule drugs that may reverse the PCa metastasis gene signature
- Selection of 11 high ranking FDA-approved drugs that are anticipated to counteract PCa metastasis genes
- Validation that 5 of 11 drugs induce apoptosis efficiently in hormone-refractory PCa cell lines
- Enhanced killing of hormone-refractory LNCaP CL1 cells by 3 of the 5 drugs as compared to parental hormone-responsive LNCaP cells
- Synergistic PCa killing by double and triple combinations of low doses of selected combinations of the 5 drugs
- Engineering of stable luciferase expressing LNCaP CL1 and LNCaP cells.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Development of luciferase expressing LNCaP CL1 and LNCaP PCa cells
List of PCa metastasis-associated genes commonly deregulated in 4 published datasets
List of high ranking small molecules apparently reversing the PCa metastasis gene signature

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and
when necessary, recommend changes on future work to better address the problem. A "so what section" which
evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

Our data provide strong evidence that gene expression profiles can be utilized to predict which drugs may be effective in killing hormone-refractory PCa cells. We have identified a metastatic PCa-specific gene signature by identifying a set of genes commonly deregulated in at least 4 published datasets of metastatic PCa. We have demonstrated that a gene expression database of FDA approved and other small molecule drugs can be exploited to identify potential anti-PCa drugs that can be tested in cell culture and eventually in animal models for preclinical validation. Since the drugs that we selected are FDA approved, clinical trials could be rapidly initiated if the animal experiments are successful. Our current data demonstrate that 5 out of 11 drugs, selected as high scorers in the Connectivity Map analysis of metastatic PCa, are indeed potent inducers of apoptosis in PCa cell lines, and 3 of them demonstrate enhanced efficacy in killing of hormone-refractory PCa cells indicating that this set of structurally unrelated drugs indeed elicit anti-PCa activity. Our future plans are to accomplish the proposed animal experiments once we have secured more resources to support this project in order to demonstrate that the selected drugs indeed have a significant impact on PCa growth and metastasis. The demonstration of anti-tumor efficacy in small animal cancer models is a key bridge from *in silico* and *in vitro* studies to human clinical testing. In particular, if a drug is already approved for another indication, proceeding with clinical testing might require only a compelling *in vitro* storyline supported by a limited number of animal studies demonstrating *in vivo* anti-tumor efficacy.

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APPENDICES: None.

SUPPORTING DATA: None
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